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REGULATION OF VERTEBRATE COLLAGENASE ACTIVITY *IN VIVO* AND *IN VITRO**

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ABSTRACT

Collagenase has been demonstrated both immunologically and enzymatically in tissue extracts from a number of vertebrates. The role of serum antiproteases, inactive enzyme precursors (zymogens) and steroid hormones in the control of collagenase activity both *in vivo* and *in vitro* is discussed.

A variety of collagenases have now been characterized from both human and animal tissues (see review, Eisen *et al.*, 1970) (1). Most of these enzymes, including human skin collagenase (2), have been isolated only from the medium of cultures of tissues suspected of producing collagenase. Initial studies of both tadpole (3) and human skin explants (4) demonstrated that the appearance of collagenase activity in the culture medium was blocked by freeze-thawing the tissue prior to culture or by the addition of puromycin to the culture medium. This indicated that *de novo* synthesis of these collagenases occurred in culture and that little or no enzyme was stored in the tissue. Alternatively it was also suggested that enzyme activation might occur by some mechanism requiring protein synthesis.

An assessment of the significance of collagenases in the *in vivo* degradation of collagen in skin and other tissues has been hampered largely by the inability to detect their presence in tissue extracts. Of the animal collagenases so far identified only those from the crustacean hepatopancreas (5) and human granulocytes (6) are extractable.

The presence of collagenase in tissue extracts was first demonstrated in human and tadpole skin (7) and subsequently in rheumatoid synovium (8) using monospecific antisera against preparations of these collagenases purified from the medium in which the tissues were cultured (9, 10). These studies demonstrated that readily detectable levels of collagenase exist *in vivo* and that the *in vivo* enzyme is immunologically identical to collagenase obtained from tissue culture. In view of these findings it seems likely that collagenase does, indeed, play an important role in collagen degradation in both normal and pathologic states. Therefore, the mechanisms for controlling collagenase biosynthesis and subsequent activity, although largely unexplored, are of considerable importance if the regulation of collagen remodeling at the molecular level is to be understood.

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Serum Antiproteases in the Control of Collagenase

Eisen *et al.* (11) showed that human serum and, in particular, the alpha globulin fraction, is capable of inhibiting both human skin and tadpole collagenases. This inhibitory capacity resides in both the alpha$_2$-macroglobulin and alpha$_1$-antitrypsin components of human serum. On the basis of these studies, it was postulated that collagenase might act freely near its site of production but that these serum antiproteases prevent its action on substrate at distant sites. Table I shows the inhibitory capability of alpha$_2$-macroglobulin and alpha$_1$-antitrypsin on human skin and rheumatoid synovial collagenases (8, 11) and demonstrates the effectiveness of small amounts of these alpha globulins in blocking enzyme activity.

Further support for a role of serum antiproteases in the control of collagenase activity is obtained when crude extracts of human skin, which have no demonstrable enzyme activity, are subjected to gel filtration on Sephadex G-150 (Fig. 1). This procedure allows partial separation of the collagenase from the serum alpha globulins, alpha$_1$-antitrypsin and alpha$_2$-macroglobulin, which are abundant in whole tissue extracts, and demonstrates that the recovery of enzymatically active collagenase is found principally in an area in which the enzyme does not overlap with these antiproteases (7). Quantitation of immunoreactive collagenase by radial diffusion (unpublished observations) and of enzymatically active collagenase by lysis of ^{14}C-labeled reconstituted collagen fibrils (Fig. 1) shows that both the immunoreactive and enzymatically active peaks are coincident, indicating that most of the collagenase isolated by the chromatography of tissue extracts is enzymatically active.

Collagenase from the peak of enzyme activity cleaves the collagen molecule at 28° C in a manner identical to that described for the enzyme isolated from the medium of human skin cultures (2, 7). Although lyophilization of the entire peak of immunoreactive collagenase virtually abolishes all enzymatic activity, immunologic analysis of this material shows that alpha$_2$-macroglobulin and alpha$_1$-antitrypsin are present in high concentration. Rechromatography of the preparation again separates enzymatically active collagenase from the alpha globulins, thus sup-

porting the concept that these serum proteins can inhibit collagenase.

The serum antiproteases alpha$_1$-antitrypsin and alpha$_2$-macroglobulin also appear to prevent detection of collagenase activity in culture medium of human skin (7) and rheumatoid synovial explants (8). Immunoreactive, but enzymatically inactive, collagenase is present in the medium shortly after cultures of these tissues are initiated.

TABLE I

Inhibition of human skin and rheumatoid synovial collagenases by serum alpha globulins

Reaction mixtures consisted of 50 μl of 0.4% ^{14}C-glycine-labeled collagen as a substrate gel, crude collagenase preparations, and variable amounts of each of the alpha globulin components in a total volume of 150–200 μl. Incubation was carried out at 37° C in a shaken water bath. Alpha$_1$-at and Alpha$_2$-M refer to Alpha$_1$-antitrypsin and Alpha$_2$-macroglobulin. (From Refs. 8 and 11.)

Enzyme source	Protein (μg)	Serum component	Protein (μg)	Inhibition (%)
Human skin	75	Alpha$_1$-at	38	18.4
	75	Alpha$_1$-at	75	49.6
	75	Alpha$_1$-at	150	68.6
	75	Alpha$_2$-M	75	51.1
	75	Alpha$_2$-M	150	69.2
	75	Alpha$_2$-M	200	80.3
Rheumatoid synovium	96	Alpha$_1$-at	74	86.4
	96	Alpha$_1$-at	148	97.0
	96	Alpha$_2$-M	235	73.4
	96	Alpha$_2$-M	940	90.0

During this period of approximately 24–48 hrs before enzymatic activity is present, the culture medium contains both alpha$_1$-antitrypsin and alpha$_2$-macroglobulin. At the time that collagenase activity first appears, these serum protease inhibitors can no longer be detected in the medium. This suggests that the failure to detect collagenase activity during the first 24–48 hrs of culture is due to the presence of these inhibitory alpha globulins which are presumably present in these tissues at the time they are placed in serum-free culture medium.

When enzymatically inactive medium of human skin or synovial explants is taken from the first day of culture and subjected to gel filtration on Sephadex G-150, collagenase activity can be detected. Demonstration of collagenase activity appears to be closely related to separation of the enzyme from alpha$_2$-macroglobulin and alpha$_1$-antitrypsin, suggesting that the absence of enzymatic activity during the initial period of tissue culture is due to the presence of these alpha globulins in the culture medium (7, 8). These *in vitro* experiments add further support for the inhibitory effect of serum antiproteases *in vivo*.

Of particular interest with respect to the role of serum alpha globulins in the control of collagenase activity are the studies of Ryan and Woessner (12). Using homogenates of involuting rat uterus taken two days after parturition these investigators have shown that not all *in vivo* collagenase is extractable and that a significant amount is bound to its connective tissue substrate. Incubation of thoroughly washed tissue pellets at neutral pH and in the presence of calcium results in the time-dependent release of hydroxyproline-containing peptides. Most of these peptides have a molecular weight less than that of collagen alpha chains indicating true lysis

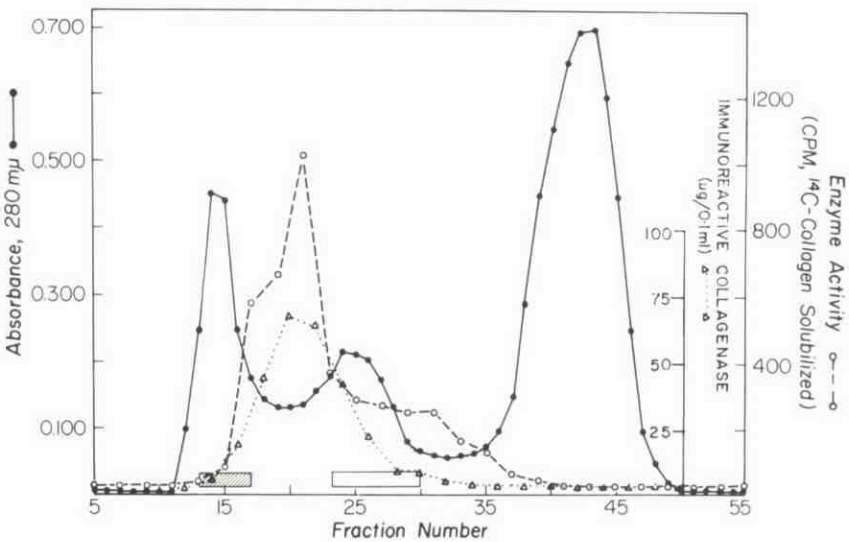

FIG. 1. Gel filtration of a fresh extract of human skin on a column (1.2 × 100 cm) of Sephadex G-150. Effluent fractions of 3.5 ml were collected at a rate of 10.5 ml/hr. ●——●, absorbance at 280 mμ; O– – –O collagenase activity; Δ· · · Δ, immunoreactive collagenase by radial diffusion. ▨, alpha$_2$-macroglobulin; ▭, alpha$_1$-antitrypsin. (From Ref. 7.)

of the collagen molecule. This tissue-bound collagenase shares many characteristics with that isolated from cultures of post partum rat uterus by Jeffrey and Gross (13).

Like extracts of human skin, the supernatant fluid of rat uterine extracts contained no enzyme activity when assayed directly. Indeed, when supernatant was added to the tissue pellet prior to incubation at 37° C, release of soluble hydroxyproline-containing material was no greater than the trypsin control, suggesting that the collagenolytic activity had been blocked. It was postulated that this inhibition of enzyme activity was due to the presence of serum inhibitors of collagenase in the supernatant fraction (12).

Similar data have been obtained using homogenates of tailfin and back skin from the metamorphosing tadpole. Table II shows that in crude extracts of tailfin, the supernatant fraction releases only 4 percent of the available radioactivity when incubated at 37° C with native ^{14}C-labeled collagen substrate. In contrast, a suspension of the tailfin tissue pellet releases approximately 29 percent of the counts, indicating that, in the actively metamorphosing tadpole tailfin, at least some collagenase is substrate-bound. The absence of significant enzyme activity in the supernatant fractions in the presence of immunoreactive collagenase (7) suggests that inhibitors of collagenase activity are present in the supernatant. An indication that little tissue remodeling occurs in the back skin of the tadpole during metamorphosis is provided by the fact that only low levels of collagenase activity are found in extracts taken from this area.

The presence of substrate-bound collagenase can be further correlated with the state of metamorphosis in the tadpole tailfin. Figure 2 shows that in tailfin tissue pellets the level of collagenase reaches a peak approximately 5 days after the initiation of metamorphosis by thyroxin. At this time about 50% of the total hydroxyproline has been lost from the tail. Continued presence of collagenase results in almost total loss of hydroxyproline and complete tail resorption. Thus in both the resorbing post partum uterus and the metamorphosing tadpole tailfin, *in vivo* collagenase appears to be of physiologic significance in collagen remodeling.

TABLE II

Collagenase activity in extracts of tailfin and backskin of metamorphosing tadpoles

Tailfins and backskins from thyroxin-treated tadpoles were extracted in 2.0 ml 0.05 M Tris-HCl (pH 7.5) with 0.005 M $CaCl_2$. Following centrifugation, tissue pellets containing approximately 10 mg of protein/ml were suspended in 1.0 ml of the same buffer. A 150 μl aliquot of an evenly dispersed suspension of the pellet or the supernatant was incubated at 37° C with ^{14}C-glycine-labeled collagen fibrils for 12 hrs. Collagen gels contained 4400 cpm. 0.01% trypsin blanks represent approximately 8% of the counts in the substrate gel.

Enzyme source	No EDTA cpm	With EDTA cpm	Net cpm	Lysis (%)
Tailfin extract				
Supernate	252	68	184	4.2
Pellet	1279	18	1261	28.7
Backskin extract				
Supernate	188	162	25	<1.0
Pellet	22	12	10	<1.0

Harper *et al.* (14) have recently reported the presence of an immunoreactive, but enzymatically inactive, precursor of tadpole collagenase in extracts of tadpole tailfin. This precursor can be converted to active collagenase by incubation with culture medium which has been previously absorbed with collagen to remove all collagenase activity. In contrast to many proenzyme-enzyme systems, this zymogen is not activated by commonly used proteolytic enzymes, such as trypsin or chymotrypsin. Activation is only accomplished by collagenase-free culture medium. The activator is heat labile, non-dialyzable and by implication a highly specific protease, which removes a portion of the zymogen molecule (molecular weight approximately 115–120,000) of about 10–15,000 Daltons. The activated tadpole enzyme is then capable of attacking both native collagen fibrils and collagen in solution, producing typical segment long spacing reaction products when examined by electron microscopy.

Although the evidence presented by these investigators points strongly to the presence of an inactive precursor of tadpole collagenase, some inconsistencies exist between the preparations of active enzyme presented in this study and those described in earlier publications from the same laboratory (15).

Initial studies on tadpole collagenase (15) indicated that the molecular weight of the amphibian enzyme was considerably less than 100,000. Harper and Gross (14, 16) have more recently presented evidence for the existence of two molecular weight species of tadpole collagenase, one of which appears to have a much higher molecule weight than that originally reported by Nagai *et al.* (15). It may be that the use of a 0–30% ammonium sulfate preparation of tadpole culture medium (in contrast to the 20–50% precipitate used by Nagai *et al.* (15)) yields a concentrated preparation of a higher molecular weight enzyme. Until further studies are done, it is difficult to determine conclusively the relationship of these two enzyme species to the zymogen. The possibility that collagenase is synthesized as a zymogen is of great interest since the presence of a highly specific activator protease, as suggested by Harper and associates (14), would provide a fundamental mechanism for the regulation of collagen degradation.

In preliminary observations on human skin collagenase, we have not, as yet, been able to dem-

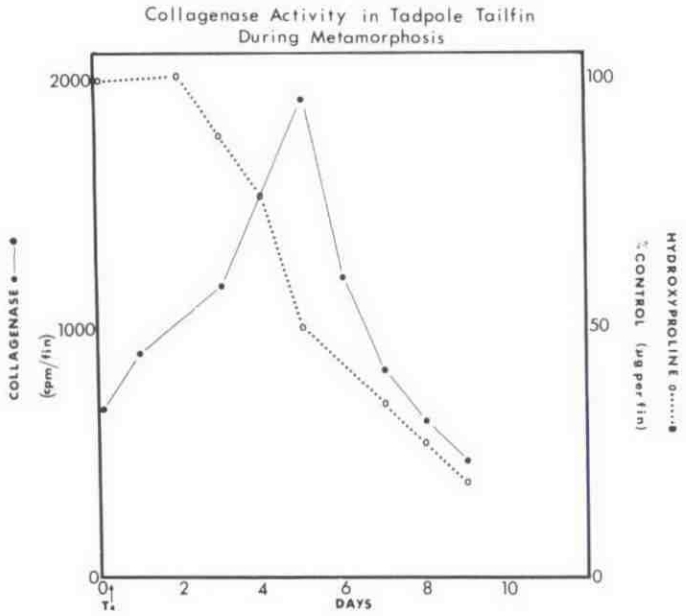

FIG. 2. Metamorphosis was induced by the addition by 10^{-6} M thyroxin to the aquarium water (arrow). Tailfins were removed daily and assayed as detailed in Table II. The collagen substrate gel contained 4780 cpm. ●——●, collagenase activity; O- - -O, total hydroxyproline per fin. On days 2, 4, 6 and 8, tailfins had lost 0, 40, 85 and 92% of their total wet weight respectively.

onstrate an inactive precursor using the classical methods of proteolytic activation of zymogens. Table III shows that attempted activation of either a crude extract of human skin or of day one culture medium from human skin explants with trypsin fails to reveal collagenase activity. The precise experiment of Harper *et al.* (14) for tadpole collagenase, utilizing collagen-absorbed culture medium as an activator, has not been performed on extracts of human skin.

It has been suggested (14) that activation of a human skin collagenase zymogen may occur during extraction and preparation of the tissue (7). This seems unlikely, since few, if any, proenzymes are activated at 0–4° C. In fact, autoactivation of most proenzymes occurs very slowly at 25° C (17, 18). It should also be noted that the activation of the tadpole collagenase zymogen by its specific protease (14) occurs very slowly even at 27° C. It would appear that extraction procedures are not responsible for activation of a human skin collagenase zymogen and that the serum antiproteases in crude tissue extracts do prevent the expression of active collagenase. Gel filtration of skin extracts (Fig. 1) supports this concept, since no areas of immunoreactive collagenase are present that lack enzymatic activity except where there is overlap with either alpha$_2$-macroglobulin or alpha$_1$-antitrypsin.

This is not quite so clear for day one culture medium of skin explants when it is subjected to gel filtration on Sephadex G-150 (7). In this case an area of immunologic reactivity that coincides with enzymatic activity can be separated from the alpha globulins, however, there is also considerable overlap of immunoreactive but enzymatically inactive material with both antiproteases. Until these alpha globulins can be completely removed from the preparation, the presence of a zymogen cannot be excluded. It is possible, then, that an inactive precursor (zymogen) may ultimately be demonstrated for human skin as well as other human and animal collagenases.

TABLE III

Attempted activation of human skin extract and day one medium by trypsin

Collagenase-trypsin mixtures were preincubated for 30 min at 37°C after which an excess of soybean trypsin inhibitor was added. The entire mixture was then transfered to ^{14}C-glycine-labeled collagen gels for 6 hrs in a shaken water bath at 37°C. Substrate gels contained 2550 cpm. Blanks represent 8.4% of the counts in the substrate gel.

Enzyme source	Enzyme protein (μg)	Trypsin protein (μg)	cpm above blank
Skin extract	0	100	203
	0	1000	326
	625	0	23
	625	100	170
	625	1000	273
Day one medium	0	100	199
	0	1000	350
	726	0	3
	726	100	144
	726	1000	210

Control of Collagenase Production in Rat Uterus

Collagen degradation in the involuting uterus constitutes one of the most rapid processes in the catabolism of this protein in mammalian physiology. It has been shown by Jeffrey and Gross (13) that cultures of rapidly resorbing rat uterine tissue secrete a specific, neutral collagenase into the culture medium. The possibility that the production of collagenase in this system is hormonally controlled has also been investigated (19, 20), and it was found that progesterone, when added to the culture medium in a concentration of 5×10^{-5} M, completely and reproducibly abolishes collagenase activity in subsequent harvests of medium. The concentration of progesterone in the tissue itself, when cultured in 5×10^{-5} M progesterone, is approximately 1×10^{-8} M, well within the physiologic limits for pregnant rat uterine tissue.

Neither estradiol nor testosterone inhibits collagenase activity in culture medium. However, the physiologically potent progesterone analogue, Provera, inhibits enzyme activity completely at 10^{-6} M. Direct addition of either progesterone or Provera to active enzyme preparations fails to inhibit collagenase activity.

When uterine tissue is cultured in the presence of progesterone, very little collagen is degraded in the tissue (Table IV). Control tissue degrades almost 90% of its collagen during the culture time (seven days), and the products of the degradation are recovered in the medium as hydroxyproline-containing peptides of low molecular weight. Table IV shows that, while tissue collagen degradation is inhibited 80% by progesterone, the loss of wet weight in progesterone-cultured tissue is only 30% less than that of control tissue. The inhibition by progesterone of collagen catabolism in the tissue, coupled with the abolition of collagenase activity, suggests that this steroid exerts a specific effect on collagen catabolism in cultured uterus.

The mechanism of action of progesterone on uterine collagenase is, at present, unknown. Progesterone itself does not inhibit the enzyme and progesterone-inhibited medium fails to inhibit active collagenase (20) suggesting that the steroid does not initiate the synthesis of an inhibitor of the enzyme. Estradiol stimulates the effect of progesterone in cultured tissue, possibly indicating that a specific progesterone receptor molecule mediates the activity of the steroid on the collagenase system.

In an *in vivo* study of rat uterine involution, Woessner (21) has found that high doses (100 μg/day) of estradiol in post-partum animals retard collagen degradation to a greater extent than uterine involution as a whole. On the other hand, Goodall (22) has found that progesterone inhibits *in vivo* involution of the rabbit uterus. Results obtained in tissue culture favor progesterone as a specific regulator of collagenase activity. It is possible that estradiol in pharmacologic doses acts to prevent collagen resorption by a different mechanism than that of progesterone.

SUMMARY

Although the precise mechanisms of the control of collagen degradation are not yet defined, a number of findings seem clear. Collagenase has been demonstrated both immunologically and enzymatically in direct extracts of a variety of tissues, thus establishing a significant *in vivo* role for collagenase in collagen catabolism (7, 8, 12, 14). In most tissues examined thus far, collagenase can be obtained from tissue extracts, but

TABLE IV

In vitro *inhibition of uterine collagen degradation by progesterone*

Uterine tissue wet weights were determined before initiation in culture and after 7 days of incubation. The final wet weight represents the weight of all the explants plus any cells or tissue debris lost from the tissue during incubation. Collagen was determined in initial tissue by hydrolyzing several randomly selected explants in 6 M HCl at 105° C for 18 h. Hydroxyproline was determined in an aliquot of the hydrolyzate. At termination of culture, all the explants were harvested, hydrolyzed and assayed for hydroxyproline. Aliquots of the pooled medium from 7 days of culture were hydrolyzed and assayed for hydroxyproline (see Ref. 20).

	Initial	Final	Difference	Change (%)
Control				
Tissue				*Loss*
Wet weight	3.69 g	1.32 g	−2.37 g	−65
Collagen	117.1 mg	16.25 mg	−100.9 mg	−86
Medium				*Recovery*
Collagen	—	105.9 mg	+105.9 mg	+105
Progesterone				
Tissue				*Loss*
Wet weight	3.69 g	2.007 g	−1.62 g	−44.7
Collagen	115.9 mg	90.6 mg	−24.3 mg	−21
Medium				*Recovery*
Collagen	—	20.01 mg	+20.01 mg	+84

in some instances, a significant fraction of the enzyme remains tightly bound to its connective tissue substrate (12).

Whether or not the control of collagenase activity in each species is identical remains to be determined. A unified concept of the control of connective tissue degradation should include the serum alpha globulins, which are capable of inhibiting collagenase activity in tissue extracts and in culture medium. It is attractive to hypothesize that their role may, indeed, be to prevent collagenase from acting at sites distant to that of enzyme production. Other mechanisms, such as the zymogen activation in the tadpole (14), may act at local levels to direct connective tissue breakdown. Although no evidence has, as yet, been found that a collagenase zymogen exists in human skin, its presence cannot be excluded. Finally, a regulatory effect of steroid hormones on collagenase from the post partum rat uterus has been found and could be operative in other physiologic, or pharmacologic situations.

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